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A Possible Structure for the Higher Oxidation State of Metmyoglobin

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In a previous paper (George & Irvine, 1954*a*) we concluded that there are two kinds of structure for the higher oxidation state of metmyoglobin (MetMb) which could explain the experimental observations. One is a radical structure formed by hydrogen atom removal from a methine carbon atom, a pyrrolic carbon atom or some other atom within the conjugated network of porphyrin ring and haemoprotein linkage; the other is a derivative of quadrivalent iron of the ferryl ion type, FeO^{2+} .

Further experiments have been undertaken to enable a choice to be made between these two structures, and the results of these are described and discussed below. The experiments fall into two sections. In the first, the hydrogen-ion changes accompanying the formation of the higher oxidation state in unbuffered solutions were measured, while in the second, the equilibrium constant for the reaction between MetMb and K_2IrCl_6 was determined under varying conditions of pH, temperature and ionic strength. The results of both classes of experiments are most simply interpreted on the basis of the ferryl ion structure.

A brief account of the pH measurements has already been published (George & Irvine, 1954*b*).

MATERIALS AND METHODS

Potassium chloroiridate. This was the same as that used previously (George & Irvine, 1954*a*). Spectrophotometric and potentiometric titrations with $\text{K}_4\text{Fe}(\text{CN})_6$ showed the sample to be 99% pure. The titrations were carried out in acid solution using glass-distilled HCl, and the solutions were made up with water distilled from dil. KMnO_4 . In this way reduction of the K_2IrCl_6 by trace reducing matter was prevented. In the experiments using unbuffered solutions, the K_2IrCl_6 was made up in CO_2 -free water as described below, and used immediately to minimize any

effect resulting from its hydrolysis. In the equilibrium experiments K_2IrCl_6 was made up in dilute glass-distilled HCl ($\sim 10^{-4}\text{M}$) to prevent hydrolysis, although, as was shown previously (George & Irvine, 1954*a*) if hydrolysis occurs to any extent, its effect is almost negligible. The strength and volumes of the buffer solutions used were such that their pH values were not affected by the acid in the K_2IrCl_6 solution.

Buffer solutions. In the experiments with K_2IrCl_6 , phosphate buffers ($\text{NaOH} + \text{NaH}_2\text{PO}_4$) were used, the ionic strengths being adjusted to the value required by the addition of AR NaCl. The pH of each solution was measured using a Cambridge pH meter calibrated with 0.05M potassium hydrogen phthalate buffer.

Metmyoglobin. MetMb was prepared and standardized as in previous papers (George & Irvine, 1952, 1953*a*).

Hydrogen peroxide. This was kindly supplied by Laporte Chemicals Ltd., as 97% (w/w) H_2O_2 , free from inhibitors. Stock solutions of approximately 0.1N were prepared by dilution, standardized against KMnO_4 , and then further diluted to the desired concentration.

Measurement of pH in unbuffered solutions. The measurements were made with a Cambridge pH-meter using a glass electrode and the usual calomel reference electrode. The instrument was calibrated with 0.05M borax solution made up in CO_2 -free-distilled water ($\text{pH} = 9.25$ at $t = 20^\circ$). Solutions of NaOH, H_2O_2 , and K_2IrCl_6 used in these experiments were also made up in CO_2 free water, and CO_2 was removed from MetMb solutions by evacuating for about 10 min. with a water pump.

In order to prevent errors during measurement due to absorption of CO_2 by the solutions, the following experimental procedure was adopted. The solution of MetMb was placed in a glass cell sealed with a Perspex cover into which were fitted the two electrodes. A steady flow of nitrogen was maintained over the surface of the liquid by means of a side arm in the cell. A Perspex screw with a small hole, fitted in the cell cover, provided an outlet for the nitrogen and, when unscrewed, enabled liquid to be pipetted into the cell.

An acid-base titration curve of MetMb, which had been thoroughly dialysed to free it from traces of salt and evacuated as described above, was obtained by measuring the pH after adding varying amounts of NaOH. 40 ml. of 10^{-4} M or 5.0×10^{-5} M MetMb were used and successive small amounts of 4×10^{-2} M-NaOH added, thus ensuring that the final volume of alkali added was small enough for changes of MetMb concentration during the titration to be negligible. Changes of pH accompanying the formation and reduction of the higher oxidation state of MetMb were then measured using a series of fresh solutions and the corresponding changes of combined H^+ were read off from the titration curve. Where the reaction took a finite time, the pH changes were followed until a steady value was obtained.

RESULTS

Hydrogen-ion changes accompanying the formation and reduction of the higher oxidation state of MetMb

Typical results from which the acid-base titration curve of MetMb was constructed are given in Table 1. The corresponding curve was found to be approximately linear between pH 5.0 and 8.0. Above this pH, the ratio of alkali added to pH change produced is not constant but increases as the pH increases, which is in accord with the high lysine content of MetMb (Tristram, 1949) increasing the buffering capacity in this region.

Measurements of pH changes accompanying the formation of the higher oxidation state with H_2O_2 and K_2IrCl_6 were carried out, in each case using a concentration of reagent just sufficient to give complete formation of the higher oxidation state. The pH remained steady for at least 5 min. after the higher oxidation state had been formed by hydrogen peroxide, and this final pH was unaffected by the addition of more peroxide. When K_2IrCl_6 was used, a small change in pH was noticed if further additions

Table 1. *Acid-base titration of MetMb at room temperature, 18°*

MetMb = 1.0×10^{-4} M (initial volume 40 ml.); NaOH = 4.15×10^{-2} M.		Concn. of NaOH added in MetMb solution (10^{-4} M)	Observed pH
Vol. NaOH added to MetMb (ml.)			
0		0	5.44
0.10		1.04	5.84
0.25		2.58	6.44
0.40		4.11	7.09
0.60		6.13	7.82
0.80		8.14	8.40
1.00		10.12	8.84
1.30		13.04	9.16
1.80		17.87	9.56
2.40		23.49	10.00

Table 2. *Hydrogen-ion changes accompanying the formation of the higher oxidation state of MetMb*

(a) *Using H_2O_2 as oxidizing agent*

(α) MetMb = 1.0×10^{-4} M; H_2O_2 = 1.8×10^{-4} M.

(β) MetMb = 5.0×10^{-5} M; H_2O_2 = 9.0×10^{-5} M.

Initial pH of MetMb	pH after addition of H_2O_2	[H^+] change interpolated from titration curve (μ M)	Ratio: H^+ produced / MetMb reacting
8.80 (α)	8.69	55.0	0.55
7.88 (α)	7.66	60.0	0.60
7.80 (α)	7.50	85.0	0.85
8.85 (β)	8.53	42.0	0.84

(b) *$Fe(CN)_6^{4-}$ added before H_2O_2*

MetMb = 1.0×10^{-4} M; H_2O_2 = 2.0×10^{-4} M.

Initial concn. of $Fe(CN)_6^{4-}$ (μ M)	Initial pH of MetMb + $Fe(CN)_6^{4-}$	pH after addition of H_2O_2	[H^+] change (μ M)	Ratio: H^+ change / MetMb reacting
100.0	7.66	7.73	-20.0	-0.20
90.0	7.82	7.89	-20.0	-0.20

(c) *Using K_2IrCl_6 as oxidizing agent*

(α) MetMb = 1.0×10^{-4} M; K_2IrCl_6 = 2.0×10^{-4} M.

(β) MetMb = 5.0×10^{-5} M; K_2IrCl_6 = 9.0×10^{-5} M.

Initial pH of MetMb	pH after addition of K_2IrCl_6	[H^+] change from titration curve (μ M)	[H^+] change after correction	Ratio: H^+ produced / MetMb reacting
7.80 (α)	7.08	200.0	160.0	1.60
8.70 (α)	8.16	215.0	170.0	1.70
9.84 (β)	9.69	75.0	60.0	1.20

were made. On the basis of this further pH change, a correction was made to the observed pH change to allow for the effect of the excess of $K_2\text{IrCl}_6$ (0.4–0.5 mole/mole MetMb) needed to produce 1 mole of the higher oxidation state: this excess is required because of the occurrence of side reactions (George & Irvine, 1954a). The results are summarized in Table 2, together with those for similar experiments in which H_2O_2 was added to a mixture of MetMb and $K_4\text{Fe}(\text{CN})_6$.

The results of reduction experiments using $K_4\text{Fe}(\text{CN})_6$ are recorded in Table 3. The higher oxidation state of MetMb (Fe_p^{IV}) was formed by adding H_2O_2 to MetMb after the pH of the MetMb solution had been adjusted to approximately 8.6 by addition of NaOH. This ensured that very little side reaction occurred (George & Irvine, 1952). In carrying out experiments at pH values other than 8.6, the initial pH of the Fe_p^{IV} solution was adjusted by the addition of HCl.

Table 3. *Hydrogen-ion changes accompanying the reduction of the higher oxidation state (Fe_p^{IV})*

$$\text{Fe}_p^{\text{IV}} = 1.0 \times 10^{-4} \text{ M}; \text{Fe}(\text{CN})_6^{4-} = 2.5 \times 10^{-4} \text{ M}.$$

Initial pH of Fe_p^{IV}	pH after reduction	[H ⁺] change from titration curve (μM)	Ratio: H ⁺ change Fe_p^{IV} reacting
8.69	9.03	–170.0	–1.70
7.89	8.51	–225.0	–2.30
7.66	8.44	–245.0	–2.50
7.50	8.27	–240.0	–2.40

The reaction between metmyoglobin and $K_2\text{IrCl}_6$

In an earlier paper (George & Irvine, 1954a) it was shown that under the appropriate conditions of pH an equilibrium is set up in this system according to the equation

Using the method of measurement described there, the equilibrium constant of reaction 1,

$$K_{\text{obs}} = [\text{Fe}_p^{\text{IV}}] [\text{IrCl}_6^{3-}] / [\text{Fe}_p^{3+}] [\text{IrCl}_6^{3-}]$$

was obtained for various conditions of pH, temperature and ionic strength. In all experiments the MetMb concentration was about $5.0 \times 10^{-5} \text{ M}$.

Variation of K_{obs} with pH. The results at two temperatures and constant ionic strength are summarized in Table 4. Inspection of the table shows that K_{obs} increases with decreasing hydrogen-ion concentration but that no linear relationship exists between K_{obs} and $1/[\text{H}^+]$ or $1/[\text{H}^+]^2$. The variation involves some more complicated dependence on $[\text{H}^+]$, greater than $1/[\text{H}^+]$ and less than $1/[\text{H}^+]^2$, in a manner reminiscent of the effect of the ionization of the haem-linked acidic group upon the

equilibrium constant for the formation of metmyoglobin-fluoride and cyanide complexes (George & Hanania, 1954a). The participation of this haem-linked ionization must be taken account of in the MetMb– $K_2\text{IrCl}_6$ reaction, and to do this the equations developed in the studies on complex formation may be used.

Table 4. *Variation of K_{obs} with pH*

(a) Temp. = $20.4^\circ \pm 0.1$; $I = 0.042$		(b) Temp. = $28.0^\circ \pm 0.2$; $I = 0.042$	
pH	K_{obs}	pH	K_{obs}
6.69	0.12 ± 0.01	6.45	0.09 ± 0.01
6.82	0.19 ± 0.02	6.66	0.20 ± 0.02
6.93	0.24 ± 0.03	6.80	0.27 ± 0.02
7.33	1.10 ± 0.10	6.89	0.40 ± 0.03
7.78	4.90 ± 0.40	7.20	0.94 ± 0.05

If it is assumed that the oxidation of MetMb by $K_2\text{IrCl}_6$ liberates 2 moles of H^+ /mole, as is indicated by the pH measurements in the preceding section, then the appropriate expression for the equilibrium constant K_T at unit $[\text{H}^+]$ is

$$K_T = K_{\text{obs}} [\text{H}^+]^2 (K_r + [\text{H}^+]) / (K_p + [\text{H}^+]), \quad (2)$$

K_r and K_p are the ionization constants of the haem-linked group in the reactant (MetMb) and in the product (the higher oxidation state) and the equilibrium constant K_T refers to the reaction in which the haem-linked group remains in its conjugate acid form throughout. Equation 2 can be rewritten

$$K_T K_p + K_T [\text{H}^+] = K_{\text{obs}} [\text{H}^+]^2 (K_r + [\text{H}^+]). \quad (3)$$

Taking $\text{p}K_r = 6.1$ at 20° and $I = 0.04$ as determined from the reaction of MetMb with fluoride (George & Hanania, 1954b), the function on the right-hand side of Eqn. 3 can be evaluated and plotted against $[\text{H}^+]$. Fig. 1a shows a typical plot for results at 20.4° and $I = 0.042$. The good linearity obtained is strong evidence for the correctness of the reaction mechanism liberating 2H^+ per mole, and on this basis values of K_T and K_p have been calculated from the slopes and intercepts of such lines. From the results plotted in Fig. 1, $K_T = (2.18 \pm 1.0) \times 10^{-14}$ and $\text{p}K_p = 7.5 \pm 0.1$. $\text{p}K_r$ is almost temperature-independent (George & Hanania, unpublished results) and from results at 28° , $I = 0.042$,

$$K_T = (3.60 \pm 0.20) \times 10^{-14}$$

and $\text{p}K_p = 7.5 \pm 0.1$ as before.

On the other hand, if it is assumed that the oxidation of MetMb by $K_2\text{IrCl}_6$ liberates 1 mole of H^+ /mole, then the equation corresponding to Eqn. 2 is

$$K_T = K_{\text{obs}} [\text{H}^+] (K_r + [\text{H}^+]) / (K_p + [\text{H}^+]),$$

and the appropriate plot to test the correctness of this assumption is of $K_{\text{obs}} [\text{H}^+] (K_r + [\text{H}^+])$ against

$[H^+]$. Similarly, on the assumption that 3 moles of H^+ are liberated/mole, the plot is of

$$K_{\text{obs}}[H^+]^3(K_r + [H^+])$$

against $[H^+]$. These give lines with a very marked curvature, as shown in Fig. 1*b* and *c*, and it can be concluded that the original assumption that 2 moles of H^+ are liberated/mole is justified.

Fig. 1. Plots of functions of K_{obs} and $[H^+]$ at 20.4° and $I=0.04$ to determine whether 1, 2 or 3 moles of H^+ are liberated per mole of MetMb in its oxidation by $K_2\text{IrCl}_6$. *a*, Plot of $K_{\text{obs}}[H^+]^2(K_r + [H^+]) \times 10^{31}$ against $[H^+]$; *b*, plot of $K_{\text{obs}}[H^+](K_r + [H^+]) \times 10^{14}$ against $[H^+]$; *c*, plot of $K_{\text{obs}}[H^+]^3(K_r + [H^+]) \times 10^{38}$ against $[H^+]$. K_r is the ionization constant of the haem-linked group in metmyoglobin, i.e. 8×10^{-7} . The good linearity of *a* is strong evidence for the liberation of 2 moles H^+ /mole.

Variation of K_{obs} with temperature. Values of K_{obs} were determined at four temperatures with both ionic strength and pH constant. The experiments were carried out using phosphate buffer of pH 6.90. This has a very small temperature coefficient so that errors arising from the change of pH with temperature, and therefore changes of K_{obs} , were negligible. A plot of $\log K_{\text{obs}}$ against $1/T^\circ K$ is shown in Fig. 2. From the slope of the graph, ΔH° for the reaction of MetMb with $K_2\text{IrCl}_6$ is found to be (10 ± 2) kcal./g.mol. This value of

ΔH° is in agreement with the value of 9.0 ± 1.0 kcal./g.mol. obtained by using the two values of K_r at 20.4 and 28° . Since the former value includes the heats of ionization of the haem-linked groups the agreement is evidence that the contribution of these ionizations to ΔH° is small.

Fig. 2. Variation of K_{obs} with temperature. Plot of $(\log_{10} K_{\text{obs}}) + 2$ against $1/T^\circ K$ at pH=6.90 and $I=0.042$.

Table 5. Variation of K_{obs} with ionic strength

Temp. = $20.2^\circ \pm 0.1$; pH = 6.95.

I	$\sqrt{I}$	K_{obs}	$(\log_{10} K_{\text{obs}}) + 1$
0.0055	0.074	0.14 ± 0.02	0.15 ± 0.05
0.012	0.110	0.16 ± 0.02	0.20 ± 0.05
0.022	0.148	0.21 ± 0.02	0.32 ± 0.04
0.042	0.205	0.27 ± 0.02	0.43 ± 0.03
0.082	0.286	0.53 ± 0.07	0.72 ± 0.05
0.162	0.403	1.35 ± 0.13	1.13 ± 0.04

Variation of K_{obs} with ionic strength. The results of these experiments are summarized in Table 5. As far as was possible the pH was kept constant at 6.95, but in cases where it was slightly different the values of the equilibrium constants were corrected on the basis of the known variation of K_{obs} with pH. The pH of buffer solutions were measured before and after the reaction. The values were the same in all cases except at the lowest ionic strength. In this case the initial pH of 6.90 fell to 6.65, and a mean value of 6.78 was taken. This fall in pH can be attributed to the buffering capacity at this low buffer concentration being exceeded by the hydrogen ions liberated during the oxidation.

A plot of $\log K_{\text{obs}}$ against $\sqrt{I}$ is shown in Fig. 3: below $\sqrt{I}=0.2$ the plot does not depart significantly from linearity with a slope of 2.0 ± 0.5 . Since K_{obs} refers to the condition of constant pH and is evaluated in terms of the concentrations of the reactants (IrCl_6^{3-} and MetMb) and of the products (IrCl_6^{3-} and the higher oxidation state) only the

charges on these four species should determine the variation of $\log K_{\text{obs}}$ with $\sqrt{I}$, provided that the simple Debye-Huckel expression for the variation of activity coefficient with ionic strength holds for these compounds in this range. Making this assumption, the slope of 2.0 corresponds to a change in effective ionic charge from +1 on MetMb to zero on the higher oxidation state.

Fig. 3. Variation of K_{obs} with ionic strength. Plot of $(\log_{10} K_{\text{obs}}) + 2$ against $\sqrt{I}$ at pH = 6.95 and temp. = 20.2° C.

The speed of the reaction. In previous experiments (George & Irvine, 1953b), which were largely carried out in solutions of pH between 8 and 9, it was noted that the oxidation of MetMb by K_2IrCl_6 is extremely rapid, with a bimolecular velocity constant greater than $10^6 \text{ l.mole}^{-1} \text{ sec}^{-1}$. In the present experiments using more acidic solutions (e.g. with pH below 7) it was observed that equilibrium was not attained 'instantaneously', but took about 10–20 sec. This suggests that the species of metmyoglobin involved in the rate-determining step is not its acidic form, but the alkaline form, in which a proton has ionized from the water molecule co-ordinated to the iron atom. The pK for this ionization is about 9 (Theorell & Ehrenberg, 1951; George & Hanania, 1952), and so at pH 7.0, 1% of the metmyoglobin would be present in the alkaline form. Thus, provided that the velocity constant was actually between 10^5 and $10^6 \text{ l.mole}^{-1} \text{ sec}^{-1}$ for reaction of the alkaline form, a time interval of the order of 10 sec. would be required to attain equilibrium at pH 7.

DISCUSSION

The results of the pH measurements in unbuffered solutions may be summarized as follows: (i) when H_2O_2 reacts with MetMb in the pH region 7.8–9.0 between 0.5 and 1.0 mole of H^+ is produced/mole of MetMb; (ii) if H_2O_2 is added to a mixture of MetMb

and ferrocyanide ion there is a very small consumption of H^+ , about 0.2 mole/mole of MetMb oxidized; (iii) in the oxidation of MetMb by K_2IrCl_6 at pH values less than 9.0, approximately 2.0 moles of H^+ are produced/mole of MetMb, whereas above pH 9.0 approximately 1.0 mole of H^+ is produced; (iv) for each mole of the higher oxidation state reduced, a little more than 2 but less than 3 moles of H^+ are consumed.

The simplest explanation of these results is afforded by the ferryl ion structure for the higher oxidation state of MetMb. If acidic MetMb is represented by the symbol $\text{Fe}_p^+\text{H}_2\text{O}$, the iron atom carrying a unit positive charge and having a water molecule attached to it in the sixth co-ordination position (Keilin & Hartree, 1949; Haurowitz, 1951), then the corresponding ferryl ion structure for the higher oxidation state would be given by Fe_pO , with zero charge on the iron atom and with an oxygen atom replacing the co-ordinated water molecule. On the basis of this structure, and taking into account the observation that a transient oxidizing entity is known to be produced in the system (George & Irvine, 1952), the reaction of H_2O_2 and MetMb in acid solution may be expressed by the following equation:

where 1 mole of H^+ is liberated/mole of MetMb reacting. In alkaline solution MetMb exists in the form Fe_pOH , the pK

having a value of about 9.0 (Theorell & Ehrenberg, 1951; George & Hanania, 1952). The reaction corresponding to 4 between H_2O_2 and alkaline MetMb should therefore yield no H^+ . Between pH 7.5 and 9.0, where both forms of MetMb are present in solution, fractional values of H^+ should be obtained. It can be seen that the results in Table 2a are in fair agreement with these expectations, provided that under these conditions the HO radical formed in reaction 4 is used up in reactions which entail no production or disappearance of H^+ .

If, however, a powerful reducing agent like the ferrocyanide ion is present initially, preferential reaction with the HO radical would be expected yielding OH^-

and, depending upon the extent to which this reaction occurred, the combination of the OH^- with H^+ produced in reaction 4 would lead to less H^+ being produced per mole of MetMb oxidized. The results in Table 2b are in accord with this. The very small consumption of H^+ under these conditions could be due to reduction of the higher oxidation state by ferrocyanide occurring to a slight extent in addition to the other reactions, for, as the results in

Table 3 show, reduction brings about the consumption of approximately 2 moles of H^+ /mole of the higher oxidation state.

In the oxidation of MetMb by K_2IrCl_6 the formation of Fe_pO from 1 mole of MetMb would involve the production of 2 moles of H^+ in acid solution, and 1 mole in alkaline solution, according to the following equations:

acid solution:

alkaline solution:

Table 2c shows that the experimental results are fairly consistent with these theoretical expectations.

The changes in H^+ -ion concentration accompanying the reduction of the higher oxidation state at various initial pH values of the solution do not give such satisfactory agreement, although the results fit into the same general pattern. In acid solution, reduction of 1 mole of Fe_pO would be expected on the basis of reaction 6 to result in the consumption of 2 moles of H^+ . The average experimental value is 2.5 moles, and the value decreases in alkaline solution, as would be expected according to reaction 7.

The pH variation of the equilibrium constant, K_{obs} , for the reaction between MetMb and K_2IrCl_6 provides more reliable evidence for the participation of 2 moles of H^+ in the oxidation reaction. Contributions to the pH variation of equilibrium constants for the reactions of myoglobin derivatives arising from the ionization of a haem-linked group, are clearly present in the myoglobin-oxygen reaction, $Mb + O_2 \rightleftharpoons MbO_2$ and in the cell reaction,

(Theorell, 1934; Taylor & Morgan, 1942). Calculations from the data for these reactions, and from more recent data on the formation of the fluoride and cyanide complexes of MetMb, show the group to have a pK of about 6.1 in MetMb (Theorell & Ehrenberg, 1951; George & Hanania, 1954b). Using this value in the appropriate equation for the variation of K_{obs} with hydrogen-ion concentration, good linearity is obtained, as shown in Fig. 1, on the basis that the oxidation liberates 2 moles of H^+ /mole MetMb oxidized, and the corresponding pK value for the linked group in the higher oxidation state is found to be 7.5. This value may be compared with that of 7.0 for the cyanide complex (George & Hanania, unpublished results), which also has a covalent structure. Corresponding plots on the basis that the oxidation liberates 1 or 3 moles of H^+ /mole do not give good straight lines, so the results do not permit these alternatives with alternative values for the pK of the linked group in the higher oxidation state. This rules out the con-

jugate acid form of the ferryl ion, Fe_p^+OH , as a possible structure for the higher oxidation state, for it would require the liberation of 1 mole of H^+ /mole in the oxidation reaction, i.e.

Thus, while there is no exact correlation in the case of the pH measurements, the results as a whole are entirely consistent with the ferryl ion structure. However, it is only the simplest structure that will account for the results. They can be explained equally well on the basis of a radical structure, but this requires the additional assumption that there is yet another ionizing group in myoglobin which participates in the reactions in a certain way. To illustrate this, acidic MetMb will be represented by the symbol $HC-Fe_p^+(H_2O)$, and the radical structure by $\cdot C-Fe_p^+(H_2O)$. Oxidation by peroxide can then be represented by reaction 8.

No liberation of H^+ occurs in this reaction which is otherwise analogous to reaction 4. But if there is an ionization of a group, say H^+Z , in the radical structure, whose pK is such that it is necessarily in its conjugate base form in the higher oxidation state, but in its conjugate acid form in MetMb (over the pH range of the present experiments), then the formation of the radical structure by peroxide would proceed according to reaction 9,

1 mole of H^+ is now liberated/mole of MetMb oxidized, exactly as in reaction 4 for the ferryl ion structure. The structure for the higher oxidation state in reaction 9 can be seen to be an isomer of the ferryl ion structure, and any other isomer would likewise fit the results with regard to hydrogen ion.

However, we could find no spectroscopic evidence over the pH range 5–7 for the presence of an ionization associated with the higher oxidation state which these isomeric structures appear to necessitate. In more acidic solutions the rapid reduction back to MetMb, which occurs spontaneously, made it impossible to tell whether there is such a group with $pK < 5$. But, although it is possible that an ionization of this kind could occur without any detectable change in spectrum (as in the case with certain haem-linked ionizations; George & Hanania, 1952, 1953), there are two lines of evidence which positively favour the ferryl ion structure. First, kinetic measurements, which will be presented in a later publication, indicate that the site of oxidation

is the iron atom; and secondly, the fact that the higher oxidation state does not apparently form its own series of complexes, although such complexes would have been expected to exist had the iron atom still been in the ferric oxidation state.

Additional support for the ferryl ion structure is provided by the variation of K_{obs} with ionic strength. As shown in Fig. 3, the slope of $\log K_{\text{obs}}$ plotted against $\sqrt{I}$ is +2.0, which is in agreement with a change in charge from +1 on MetMb to zero on the higher oxidation state, like that obtained for the formation of metmyoglobin-fluoride (George & Hanania, 1954b), viz.

There is, however, one special case of the radical structure in reaction 9 which would lead to this same result, namely identifying the group H^+Z with the iron atom and its co-ordinated water molecule, i.e.

Furthermore, the inability of the higher oxidation state to form its own complexes could be attributed to the OH group in this structure being strongly bound. On the other hand, this structure is not in accord with the kinetic evidence showing that the site of oxidation is the iron atom itself. And another reason for preferring the ferryl ion structure, although it is purely qualitative, is that many free radicals readily combine with molecular oxygen resulting in irreversible oxidation with the formation of carbonyl and hydroxyl groups, and often, in addition, in degradation of the molecule through simple bond breaking or ring fission (George & Walsh, 1946). The observation that the higher oxidation state of MetMb is unaffected by oxygen (George & Irvine, 1954a, c), argues for a structure of a fundamentally different kind, and the oxidation states containing bonded O-atoms, familiar in inorganic chemistry in the ions VO^{2+} , UO_2^{2+} , MnO_4^- , etc., fulfil this requirement. Thus, although the ferryl ion structure does not provide a unique explanation for the results, it may be accepted and the higher oxidation state accordingly named 'ferrylmyoglobin', until there is good reason to prefer one of the isomeric structures.

From the pK values for the linked ionizing group in metmyoglobin and ferrylmyoglobin, together with K_{obs} and E'_0 for the $\text{IrCl}_6^{3-}/\text{IrCl}_6^{2-}$ couple, the pH variation of the oxidation-reduction potential for the $\text{Fe}_p\text{O}/\text{Fe}_p^+(\text{H}_2\text{O})$ couple can now be calcu-

lated. The results are plotted in Fig. 4. This curve is somewhat different from that published previously (George & Irvine, 1954a) for the following reasons: first, in constructing the previous curve, E'_0 at pH 6.82 was calculated using the value $E_0 = 1.017\text{v}$ obtained by Dwyer, McKenzie & Nyholm (1944) for the $\text{IrCl}_6^{3-}/\text{IrCl}_6^{2-}$ couple. We have recently re-determined it and found the value of 0.898v at $I = 0.04$ and $T = 20^\circ$ to be a better one; secondly, the

Fig. 4. Calculated variation of E'_0 with pH, at 20.4° and $I = 0.042$, for the couple involving the higher oxidation stage of metmyoglobin. *a*, Point of inflexion due to haem-linked ionizations; *b*, the line changes slope due to the ionization of the iron-bound water molecule in metmyoglobin, $\text{Fe}_p^+(\text{H}_2\text{O}) \rightleftharpoons \text{Fe}_p\text{OH} + \text{H}^+$.

contribution made by the ionization of the haem-linked group over the pH range 6.5–8.0 has now been taken into account; thirdly, the method used previously for fixing the portion of the curve above pH 8.0 by means of pH thresholds of oxidation of MetMb or reduction of the higher state was not precise, since it did not allow for the relative concentrations of the reactants. In Fig. 4, between pH 8.0 and 9.0, E'_0 varies according to 2H^+ in the standard equation for the couple, and at pH 9.0, the pK of MetMb, there is a change in slope of the curve corresponding to the change from 2H^+ to 1H^+ in the equation,

The portion of the curve above pH 9.0 is not quite so reliable, because it is not possible to assess E'_0 accurately in this region since the pK of MetMb rises slightly as the pH is increased beyond 9.6 (George & Hanania, unpublished results).

The measurement of the variation of K_{obs} with temperature for the reaction of MetMb with K_2IrCl_6 leads to a value of (10.0 ± 2.0) kcal./g.mol. for ΔH° . From the values of K_T at 20.4 and 28°, and from the variation of K_{obs} with ionic strength, K_T is estimated to be 2.88×10^{-14} at 25° and zero ionic strength. Using the equations

$$\Delta G^\circ = -RT \ln K \quad \text{and} \quad \Delta G^\circ = \Delta H^\circ - T\Delta S^\circ,$$

ΔS° for the reaction is found to be -30 e.u. From this value we can calculate the difference between the standard partial molal entropies, $\bar{S}^\circ$ (i.e. standard entropies in solution), of ferrylmyoglobin and metmyoglobin and compare it with the corresponding differences between the partial molal entropies of metmyoglobin complexes and metmyoglobin, and similar data for ferric ion complexes.

For this purpose, the reaction between MetMb and K_2IrCl_6 must be written out in full, in the form,

and the entropy change of -30 e.u. is seen to be made up as follows

$$-30 = \bar{S}^\circ_{\text{Fe}_p\text{O}} + \bar{S}^\circ_{\text{IrCl}_6^{3-}} - 2\bar{S}^\circ_{\text{H}^+} - \bar{S}^\circ_{\text{Fe}_p^+} - \bar{S}^\circ_{\text{H}_2\text{O}} - \bar{S}^\circ_{\text{IrCl}_6^{2-}}.$$

From the variation of E_0 with temperature for the cell reaction

(Latimer, 1952). This suggests that desolvation effects predominate in the formation of these inorganic complexes and that there are compensating changes within the configuration of the various complexes so that the $\bar{S}^\circ$ values are approximately the same in spite of their different structures, e.g. FeOH^{2+} , FeF^{2+} , FeCNS^{2+} , etc. In the case of the metmyoglobin complexes the differences between the $\bar{S}^\circ$ values are again approximately constant but the values are much smaller which suggests that desolvation is a far less important factor. In charge type ferrylmyoglobin is identical with the hydroxyl, fluoride and cyanide complexes, and its magnetic susceptibility indicates covalent bond formation as in the cyanide complex (Theorell & Ehrenberg, 1952). The difference in $\bar{S}^\circ$ of +6.7 e.u., evaluated on the assumption that the higher oxidation state has the ferryl ion structure, is very close to the value of +4.2 e.u. for the cyanide complex, and not very different from the values for the fluoride and hydroxyl complexes in which the bonding is predominantly ionic.

Although this agreement may be fortuitous, it can be concluded on the basis of the thermodynamic data at present available that the magnitude of the difference between the partial molal entropies of metmyoglobin and its higher oxidation state is quite consistent with the higher oxidation state possessing the ferryl ion structure.

Table 6. *Standard entropies of myoglobin and ferric ion complexes*

Myoglobin	Ferric ion
$\bar{S}^\circ_{\text{Fe}_p\text{O}} - \bar{S}^\circ_{\text{Fe}_p^+} = +6.7$ e.u.	$\bar{S}^\circ_{\text{FeOH}^{2+}} - \bar{S}^\circ_{\text{Fe}^{3+}} = +47.5$ e.u.
$\bar{S}^\circ_{\text{Fe}_p\text{CN}} - \bar{S}^\circ_{\text{Fe}_p^+} = +4.2$ e.u.	$\bar{S}^\circ_{\text{FeF}^{2+}} - \bar{S}^\circ_{\text{Fe}^{3+}} = +46.7$ e.u.
$\bar{S}^\circ_{\text{Fe}_p\text{OH}} - \bar{S}^\circ_{\text{Fe}_p^+} = -5.1$ e.u.	$\bar{S}^\circ_{\text{FeCl}^{2+}} - \bar{S}^\circ_{\text{Fe}^{3+}} = +48.2$ e.u.
$\bar{S}^\circ_{\text{Fe}_p\text{F}} - \bar{S}^\circ_{\text{Fe}_p^+} = -0.5$ e.u.	$\bar{S}^\circ_{\text{FeBr}^{2+}} - \bar{S}^\circ_{\text{Fe}^{3+}} = +42.3$ e.u.
	$\bar{S}^\circ_{\text{FeCNS}^{2+}} - \bar{S}^\circ_{\text{Fe}^{3+}} = +44$ e.u.

we have found $\bar{S}^\circ_{\text{IrCl}_6^{2-}} - \bar{S}^\circ_{\text{IrCl}_6^{3-}}$ to be -20 e.u., and so since $\bar{S}^\circ_{\text{H}^+}$ is by convention zero, and $\bar{S}^\circ_{\text{H}_2\text{O}}$ is 16.7 e.u. (Latimer, 1952), it follows that

$$\bar{S}^\circ_{\text{Fe}_p\text{O}} - \bar{S}^\circ_{\text{Fe}_p^+} = +6.7 \text{ e.u.}$$

Corresponding values for the hydroxyl, fluoride and cyanide complexes of metmyoglobin are listed in Table 6, together with values for several complexes of the ferric ion (George & Hanania, 1952 and unpublished results; Betts & Dainton, 1953; Uri, 1952).

For the inorganic complexes the differences in $\bar{S}^\circ$ values are large and positive, which is in accord with expectation since the more highly charged ion has the water molecules of solvation more tightly bound around it. These differences are in fact approximately the same as the difference between $\bar{S}^\circ$ for ferrous and ferric ion, i.e.

$$-27.1 - (-70.1) = +43.0 \text{ e.u.}$$

SUMMARY

1. In unbuffered solutions, measurements have been made of the pH changes which accompany the formation and the reduction of the higher oxidation state of metmyoglobin. For each mole of MetMb oxidized by H_2O_2 in the pH region 7.5 to 9.0, between 0.5 and 1.0 mole of H^+ is produced. In the formation of the higher oxidation state by K_2IrCl_6 about 2 moles of H^+ are produced in acid solution, and about 1 mole in alkaline/mole of MetMb. The reduction of the higher oxidation state by $\text{K}_4\text{Fe}(\text{CN})_6$ yields slightly more than 2 but less than 3 moles of H^+ .

2. The equilibrium constant (K_{obs}) for the reaction between MetMb and K_2IrCl_6 has been measured under various conditions of pH, temperature and ionic strength. It was noted that in the

more acidic solutions, with $\text{pH} < 7.0$, a few seconds elapsed before equilibrium was attained. Taking into account the ionization of the haem-linked group on MetMb and the higher oxidation state, the variation of K_{obs} with pH is shown to confirm the conclusion that 2 moles of H^+ are liberated/mole of acidic MetMb. Using 6.1 for the pK of the group in MetMb as established in other studies, the results give a pK of 7.5 for the group in the higher oxidation state at 20° and $I = 0.04$.

3. The variation of K_{obs} with temperature gives $\Delta H^\circ = 10.0 \pm 2.0$ kcal./g.mol.: if the ionization of the haem-linked group is allowed for, the value 9.0 ± 1.0 kcal./g.mol. is obtained.

4. The dependence of K_{obs} on ionic strength is in accord with a change in charge from +1 on MetMb to zero on the higher oxidation state.

5. The results are shown to favour the ferryl ion structure, or an isomer of this structure, for the higher oxidation state. The isomeric structures would, in general, require the presence of another ionizing group in myoglobin, but no evidence for such an ionization could be found. With other direct evidence favouring the ferryl ion structure this is to be preferred, and the higher oxidation state may provisionally be named ferrylmyoglobin.

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Tissue Fractionation Studies

6. INTRACELLULAR DISTRIBUTION PATTERNS OF ENZYMES IN RAT-LIVER TISSUE*

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The finding that the acid phosphatase of rat liver is enclosed within a special type of cytoplasmic granules, with sedimentation properties intermediate between those of mitochondria and microsomes, has led to the development of a new scheme of fractionation, whereby enzymes attached to these granules can be readily identified (Appelmans, Wattiaux & Duve, 1955). This scheme has been applied in the present work to the study of the

following enzymes, previously shown to resemble acid phosphatase in being unequally distributed between mitochondria and microsomes: DPNH-cytochrome *c* reductase (Hogeboom, 1949; Hogeboom & Schneider, 1950a; Strittmatter & Ball, 1954), TPNH-cytochrome *c* reductase (Hogeboom & Schneider, 1950b), β -glucuronidase (Walker, 1952), cathepsin (Maver & Greco, 1951), ribonuclease (Schneider & Hogeboom, 1952b; Pirotte & Desreux, 1952), deoxyribonuclease (Schneider & Hogeboom, 1952b; Kuff & Schneider, 1954), uricase (Schein, Podber & Novikoff, 1951; Schneider & Hogeboom, 1952a; Novikoff, Podber, Ryan & Noe, 1953; Kuff & Schneider, 1954) and fumarase (Kuff, 1954). In addition, the distributions of

* Part 5: Appelmans, Wattiaux & Duve (1955).

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